TISSUE CULTURE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF NORTH AMERICAN GINSENG (*PANAX QUINQUEFOLIUM* L.)

by

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Tissue Culture and Agrobacterium - mediated Transformation of North American Ginseng (Panax quinquefolium L.)

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Abstract

Somatic embryogenesis in *Panax quinquefolium* L. was established from three explant sources: root, leaves (seedlings and mature), and epicotyls. Roots and mature leaves were obtained from field-grown plants, while seedling leaves and epicotyls were obtained from lab-grown seeds. The optimal growth regulators for callus growth of root explants on Murashige and Skoog (MS) medium were dicamba/kinetin (9.0/5.0 μM), whereas for somatic embryo development, dicamba (9.0 μM) was optimal (15.6%). For epicotyl explants, callus growth and somatic embryo formation were optimal on MS medium with dicamba/kinetin (9.0/5.0 μM). For leaf explants, however, this combination of dicamba/kinetin was optimal solely for callus growth. The highest rate of somatic embryo formation (40%) was achieved within a 3 month period with seedling leaf-derived calluses plated onto MS medium with NAA/2,4-D (10.0/9.0 μM). Somatic embryo formation from mature leaf-derived calluses plated onto MS medium with NAA/2,4-D (10.0/9.0 μM) was obtained 7 months after the intial culture, at a frequency of 20%.

Plantlet recovery was achieved through a two-stage process, involving the elongation of the shoot axis, followed by the formation of roots. Addition of activated charcoal (1%) to the embryo germination medium had a positive effect on root development. The highest percentage of shoot axis elongation from somatic embryos was 70% on half-strength MS salts with charcoal (1%), whereas plantlet formation from somatic embryos was 90% on MS medium containing NAA/2,4-D (5.0/4.5 μM) and charcoal (1%).

Preculture of root explants for 1 week before infecting with *Agrobacterium tumefaciens* disarmed strain EHA 105 containing plasmid p1779C had a positive effect on the percentage of putatively transformed calluses (15.5%). For leaf explants infected with *A.-tumefaciens* disarmed strain EHA 105 containing plasmid pGA492-

CHN, the highest percentage of explant survival (28%) was obtained after 3 days of cocultivation on medium with NAA/2,4-D (10.0/9.0 μM), without preculture. The regeneration capacity of these putatively transformed calluses has not been determined.

Dedication

To
Randy, Keizar
Papa, Mama, Harold
for their encouragement, patience and everlasting love

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CHAPTER 1

Introduction

1.1 The genus *Panax*

The genus *Panax* belongs to the class Dicotyledonae and is a member of the family Araliaceae. This family consists of 65 genera and approximately 800 species (Lawrence 1960), which are distributed primarily throughout the Indo-Malayan region and tropical America. *Panax* is the major genus which is commonly found in North America (Proctor and Bailey 1987).

Ginseng, a fleshy-rooted herbaceous perennial plant, belongs to the genus *Panax*. An extract of the fleshy root of ginseng has been used traditionally in Asia for geriatric, tonic, stomach and aphrodisiac treatment (Choi 1988). Gradually, ginseng has increased in popularity not only in Asian countries but throughout the world. Increased performance during fatigue, stimulation of the central nervous system during depression, and improvement in skin moisture are some of the main claims for ginseng action (Kim 1978). It is now believed that ginsenosides are the constituents that are primarily responsible for the pharmacological action of ginseng. Extracts containing these substances are believed to act as adaptogens (Proctor and Bailey 1987).

There are seven *Panax* species in North America and Asia according to the classification of Hu (1976) and Lewis (1979):

1. *Panax quinquefolium* L., also called North American or Canadian ginseng, found in North America.
2. *Panax trifolium* L., also called dwarf ginseng or ground nut, found in North America.
3. *Panax ginseng* C.A. Meyer, also called Oriental ginseng, found in North China, Korea, Manchuria and North Japan.

4. *Panax pseudoginseng*, also called Sanchi ginseng, found in China

5. *Panax bipinnatifidum* Seem., also called feather leaf or bamboo ginseng, found in China.

6. *Panax japonicum* C.A. Meyer, also called bamboo ginseng, found in China.

7. *Panax major* (Burk.) Ting., also called big leaf sanchi, found in China.

American ginseng and Oriental ginseng are the major commercial species cultivated at present .

1.2 Ginseng cultivation

In nature, ginseng is a plant that requires a minimum of 70% shading for optimal growth. Cultivation of ginseng was originally initiated by planting wild roots and collecting seeds from wild plants. At present, all seeds produced by these domesticated plants are used for the next planting season. Stratification of ginseng seeds with a fluctuating cool-warm-cool temperature regime over a 18-20 month period is a requirement since the embryo is still immature when the seeds are harvested (Proctor and Bailey 1987).

Currently, there are two ways of growing ginseng: natural and artificial shade grown. Artificial shade grown is by far the most common method used worldwide for ginseng cultivation. Both of these cultural methods have several common features, such as shade (natural or artificial), mulch (natural leaf fall, applied straw or other suitable material) and raised beds for growing the ginseng plants (Proctor and Bailey 1987).

The major method of ginseng propagation is through seed. Ginseng plants are obtained either by direct seeding into the field, or by seeding into a nursery bed and transplanting after 1 year of growth. The ginseng seedling consists of a single stalk with three leaflets and root. In the first year, this leaf and stalk will be 5-10 cm high and the root will be less than 1 gram in fresh weight. The aerial portion of the ginseng plant dies back annually at the end of the season. American ginseng resumes foliar growth in the middle of April or early May. Normally, the flower bud and leaves emerge simultaneously (Proctor and Bailey 1987; Hu et al. 1980). After 4 years of growth, ginseng foliage will reach up to 40-60 cm in height and the root may be up to 200 grams in fresh weight, although the average is about 14 grams (Oliver 1996). A mature ginseng plant has several whorled leaves at the top. This whorled leaf is called a prong, and consists of a petiole with three to five leaflets. The first seed production occurs after 2-4 years of growth, and each mature berry fruit yields two or three creamy white to yellow seeds (Proctor and Bailey 1987).

Plant diseases are the primary problem faced by ginseng producers. Ginseng diseases range from those that cause premature death of plants to those that reduce the quality of roots. The average yield of ginseng can be reduced up to 60% by these problems (Ohh 1981; Proctor and Bailey 1987). The most commonly encountered fungi are: *Rhizoctonia* and *Pythium* spp., *Phytophthora cactorum*, *Cylindrocarpon* sp., and *Alternaria panax* (Oliver 1996).

There are no ginseng cultivars reported to date. Selection of superior lines has been done solely by the appearence of ginseng plants in the field. A conventional ginseng breeding program has several associated problems, including the long reproductive cycle, 18-20 months of seed dormancy, and difficulties in interspecific cross breeding (Proctor and Bailey 1987). A potential solution to these problems is to use tissue culture techniques. With these techniques, a superior line of ginseng could be propagated in higher numbers and within a shorter period of time than by

natural processes. Moreover, the ability to regenerate plants from isolated tissues or organs *in vitro* would provide a vehicle for plant transformation,germplasm preservation, and clonal propagation for uniformity of traits.

1.3 An overview of plant tissue culture

1.3.1 History

The history of plant tissue culture dates back approximately 240 years to the discovery of callus formation by Duhamel. His discovery that a small ring of cortex from an elm tree had the ability to develop into undifferentiated tissues and new buds, is believed to be the beginning of tissue culture (Gautheret 1985). This discovery was followed by the general cell theory of Schleiden (1838) and Schwann (1839), who proposed that each living cell of a multicellular organism has the ability to develop independently if proper enviromental conditions are provided. A cell which is capable of developing and subsequently regenerating a copy of the original organism is referred to as a totipotent cell (Gautheret 1985).

Subsequent experiments by several researchers using fragments of tissues isolated from higher plants have proven this theory experimentally. Formation of callus at wound sites of isolated stem fragments and root slices has been observed by several researchers. Up to this point, there had been no nutrients used in any of the experiments. Haberlandt was the first researcher to propose the concept of cell culture. In his experiments, he attempted to cultivate isolated plant cells *in vitro* on an artificial medium. From these experiments, Haberlandt proposed the cell division theory. According to this theory, cell division was regulated by two hormones: leptohormone, which was associated with vascular tissue and a wound hormone, which was released by injured cells (Dodds and Roberts 1985; Gautheret 1985).

The first plant tissue cultures were established by Gautheret and Nobecourt using cambial tissues of carrot as explants. In their experiments, they demonstrated the unlimited potential growth of a callus culture; however, plant regeneration was not achieved. Subsequent research, therefore, was focused on determining the proper conditions for further growth of these undifferentiated calli. One of the most significant steps leading to the establishment of further growth of undifferentiated cells was the discovery of the nutritional quality of coconut milk. The combination of coconut milk with 2,4-D exhibited a remarkable effect on the proliferation of cultured carrot and potato tissues. Zeatin riboside was later confirmed to be partly responsible for the stimulatory effect of coconut milk (Dodds and Roberts 1985).

The discovery of the stimulatory effect of coconut milk led to the discovery of cytokinin by Skoog and his collaborators in 1944 (Gautheret 1985). Later, in 1957, Skoog and Miller proposed that the initiation of shoots and roots in cultured callus could be regulated by varying the ratio of auxin and cytokinin in the medium. However, Thorpe (1980) concluded that the determination of organ formation in tissue culture is a more complex process and therefore cannot be restricted to hormonal balances only. He asserted that other factors such as plant species, tissue type, light, temperature, mineral composition of the nutrient solution and sugar concentration also have important roles in determining the regeneration process.

1.3.2 Regeneration of plants through tissue culture

The choice of explants is a critical factor in establishing a successful regeneration system *in vitro*. In general, explants must originate from a healthy and vigorous plant. According to the totipotency theory, all cells are capable of regenerating into a complete plant if optimal environmental conditions are provided. However, it was observed that immature tissues and organs are morphogenically more responsive to regeneration than mature tissues. Shoot tips, axillary buds,

immature embryos, leaf and stem segments are examples of good explant sources (Rao 1987).

Surfaces of explants usually carry several microbial contaminants. Therefore, before being plated onto the medium, these explants are first surface sterilized. Hypochlorite solution (sodium and calcium) and ethyl or isopropyl alcohol are the most common compounds used for explant sterilization. Generally, these sterilizing agents can be used individually or together as a two-stage disinfection. Since these compound are also toxic to the explants, the concentration and the duration of the sterilization procedures varies according to the type of explants. In general, rinsing with alcohol for a few seconds and subsequently dipping into 0.3-0.6% sodium hypochlorite solution for approximately 15-30 min, are sufficient to decontaminate most explant tissues (Bhojwani and Razdan 1983). The addition of a few drops of a surfactant, such as Triton-X or Tween-80, to the hypochlorite or ethyl/isopropyl alcohol solution has proven to be effective in enhancing sterilization efficiency (Bhojwani and Razdan 1983; Dodds and Roberts 1985).

In nature, plants obtain nutrients to support their growth from the soil, water and air. Generally, the requirements of plant tissues grown *in vitro* are similar to those grown in soil. However, since only small pieces of plant tissues or organs are used as a starting material, these isolated tissues or organs do not have the capability to provide their own carbohydrates, vitamins and other growth substances. Therefore, these components have to be provided artificially (Akins and Vasil 1985; Dodds and Roberst 1985). A typical tissue culture medium consists of inorganic and organic nutrients, carbon source, iron, vitamins and plant growth regulators. In certain instances, natural products such as coconut milk, fruit pulp and juice or other plant extracts and yeast extract may also be supplemented to the medium (Dodds and Roberts 1985).

Nitrogen in tissue culture medium is usually present as either nitrate or ammonium, or as a combination of these ions. Magnesium and sulfur requirements are provided generally by $MgSO_4 \cdot 7H_2O$ and phosphorus as either $NaH_2PO_4 \cdot H_2O$ or KH_2PO_4. Potassium is usually provided as KCl, KNO_3 or KH_2PO_4 and calcium as either $CaCl_2 \cdot 2H_2O$ or $Ca(NO_3)_2 \cdot 4H_2O$. Micronutrients like Fe, Mn, B, Zn, Mo, Cu, I and Co are generally included in plant tissue culture media, whereas Al and Ni are used only in special circumstances (Dodds and Roberts 1985; Akins and Vasil 1985).

Sucrose, at a concentration of 2-3%, is the most commonly used carbohydrate in plant tisue culture media since nearly all cultures showed optimal growth in response to these concentrations (Dodds and Roberts, 1985). Other disaccharides such as maltose, lactose, cellobiose, melibiose and trehalose have also been used in several instances, depending on the plant species (Akins and Vasil 1985).

Myo-inositol, thiamine, pyridoxine, nicotinic acid and the amino acid glycine are the most common vitamins employed in tissue culture media. Although myo-inositol is a carbohydrate,it is usually grouped with the vitamins in tissue culture media. Myo-inositol has proven necessary for most of experiments, and is included in most media at 100 mg/l. Thiamine, pyridoxine, nicotinic acid and glycine are used less often than myo-inositol (Akins and Vasil 1985). Several basic tissue culture media containing different concentrations of macro/micro nutrients and vitamins such as White (1943), Heller (1953), Murashige and Skoog (1962), Linsmaier and Skoog (1965), B5 (Gamborg et al. 1968), Nitsch and Nitsch (1969) and Schenk and Hildebrandt (1972) have been developed by various researchers, and were named mostly after the investigators.

Auxin and cytokinin are the most common growth regulators required in tissue culture. In nature, auxin is generally involved with elongation of stems and internodes, tropism, apical dominance, abscision and rooting. In tissue culture, the use of auxin is associated with cell division and root differentiation (Bhojwani and

Razdan 1983). The synthetic auxins, naphthaleneacetic acid (NAA) and 2,4-D are the types of auxin most frequently used in tissue culture. Other synthetic auxins, such as trichlorophenoxyacetic acid (2,4,5-T), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2-methoxy-3,6-dichlorobenzoic acid (dicamba) and 4-amino-3,5,6-trichloropicolinic acid (picloram) have also being employed in several instances (Akins and Vasil 1985).

In nature, cytokinins are generally associated with cell division, modification of apical dominance and shoot differentiation. The application of cytokinins in tissue culture is primarily related to cell division and differentiation of adventitious shoots from callus and shoot proliferation (Bhojwani and Razdan 1983). Kinetin (6-furfurylamino purine), 6-benzylaminopurine (BAP), and 6-γ,γ-dimethylallylaminopurine (2i-P) are most often employed in tissue culture as cytokinins.

Gibberellins (GAs), abscisic acid (ABA) and ethylene are three other classes of growth regulators that also exert some control on morphogenesis. However, compared to auxin and cytokinin, gibberellin, abscisic acid and ethylene are used less often (Akins and Vasil 1985). The supplement of abscisic acid to the medium is usually associated with its effect in causing maturation of the somatic embryos. One of the important factors influencing germination and plantlet growth *in vitro* is the maturation process of somatic embryos (Lelu et al. 1994 a,b). A phenomenon called precocious germination, due to the fast transition from the late embryogeny stage to the germination stage without going into a phase of developmental arrest, is sometimes observed in somatic embryogenesis. As a result, the germinating somatic embryos may possess several abnormalities such as the lack of root or shoot meristem (von Aderkas et al. 1991; Lelu et al. 1994 a,b). Addition of abscisic acid (60 μM) to the media has proven to reduce this precocious germination in hybrid

larch (*Larix* x *leptoeuropaea*) and increased the germination and plantlet development rate from 0 to 93% and 80%, respectively (Lelu et al. 1994 a).

Addition of silver nitrate, primarily in the form of $AgNO_3$ at 1 to 10 mg/l has proven useful for enhancing plantlet regeneration in several species, such as muskmelon (*Cucumis melo)* (Roustan et al. 1992) and corn (*Zea mays)* (Songstad et al. 1991). The role of $AgNO_3$ in increasing plantlet regeneration is related to its ability to inhibit the negative effect of ethylene, which is occasionally produced by cultured tissues (Beyer 1976).

Thiadizuron was initially developed as a cotton defoliant in 1976 (Arndt et al. 1976). Further studies suggested that this compound has a strong cytokinin-like activity (Mok et al. 1982). Since then, thiadizuron has been used in tissue culture for its positive effect, including inducing somatic embryogenesis, adventitious shoot formation and axillary shoot proliferation. For example, in peanut (*Arachis hypogea*), exposure of the hypocotyl region for 1 week to 10 μM of thiadizuron stimulated the initiation of adventitious shoots that subsequently developed into plantlets. Its effect in tissue culture has been associated with its ability to stimulate rapid conversion of ribonucleotides to ribonucleosides (Capelle et al. 1983) and the synthesis and accumulation of purine cytokinins (Thomas and Katterman 1986).

Growth and development in tissue culture are also affected by the following factors: pH of the medium, humidity, light, temperature and the physical form of the medium (Rao 1987). In general, the culture room is usually maintained between 20 and 30 ^{o}C (Rao 1987). The effect of relative humidity is rarely investigated; however, if the humidity in the culture room is less that 50%, it will cause the medium to dry rapidly (Bhojwani and Razdan 1983). Light supply in the culture room is usually provided by fluorescent lamps with intensity less than 1000 lux and the pH of the medium ranges from 5.0 to 6.0 (Bhojwani and Razdan 1983). The optimal

combinations of these environmental factors must be determined for each species and the intent of the research.

1.3.3 Modes of plant regeneration *in vitro*

Regeneration of plants through tissue culture is obtained through three processes: organogenesis, somatic embryogenesis, and enhancement of axillary branching.

Roots, shoots and flowers are examples of the types of organs which can be initiated from explants in tissue culture. Organogenesis can be direct or indirect. Direct organogenesis is the direct differentiation of organs, such as shoot buds from explants. The formation of shoots and roots via callus formation of explants is termed indirect organogenesis (Rao 1987). The underlying factors involved in organogenesis are difficult to define generally, since the stimuli may involve a combination of medium component, endogenous compounds produced by the culture itself, and substances carried by the explants (Thomas and Davey 1957). According to Rao (1987), explant size, physiological age of the explant, and seasonal variation, are several factors that are known to affect the organogenetic response of any particular explant (Rao 1987). Smaller explants have been observed to have reduced regenerative ability compared to larger explants. The physiological age of explants can also influence the type of organogenesis. For example, in *Escheveria,* young leaf explants produced only roots, whereas older leaves regenerated only shoot buds and leaves of medium age produced both roots and shoots (Rao 1987; Raju and Mann 1970). The effect of seasonal variation has been observed with potatoes. Explants initiated from plants obtained during December - April were highly tuberogenic when compared to those obtained from plants during the rest of the season (Fellenberg 1963).

Somatic embryogenesis is the *in vitro* production of somatic embryos from isolated somatic cells. In tissue culture, somatic embryos may be initiated from different types of cells: vegetative cells of mature plants, reproductive tissues other than zygotes and from hypocotyls and cotyledons of seedlings, or from young plantlets (Dodds and Roberts 1985). Somatic embryogenesis may occur either directly from single cells called "preembryonic determined cells" or indirectly through an intervening callus phase. In the latter case, embryos originate from "induced embryogenic cells" (Dodds and Roberts 1985; Rao 1987). Somatic embryogenesis has a distinctive sequential stages of embryo formation, and are termed globular, heart shape, and torpedo shape. According to Kohlenbach (1978), the embryo formation is regulated by two important events: 1) the induction of cytodifferentiation of the proembryoid cells, and 2) the unfolding of the developmental sequence by these proembryoid cells. While any given cell could differentiate into an embryogenic cell, its further development is dependent on the chemical balance present in the medium. As a result, two types of media are used in sequence for somatic embryogenesis. The first medium is for initiating embryogenic cells and the second medium is for the subsequent development of these cells into embryos. Generally, the first medium contains auxin, whereas the second medium will contain either a lower concentration of the same auxin, a reduced level of a different auxin or no auxin at all. In some cases, both embryo initiation and maturation may occur in the first medium. The second medium is then necessary for plantlet development (Ammirato 1983).

Activated charcoal supplemented to the medium has been observed to have a stimulatory effect on somatic embryogenesis in some plants, for example carrot and English Ivy (Drew 1979; Banks 1979). The precise role of activated charcoal in embryogenesis remains unknown. However, evidence suggests that charcoal may

absorb a variety of inhibitory substances as well as growth promoters in the medium (Ammirato 1983).

Some plants naturally have the ability to produce adventitious buds from organs such as root, leaf and bulbs. Through tissue culture, the rate of adventitious bud development can be enhanced, as in the case of begonia (Rao 1987; Reuter and Bhandari 1981).

1.4 Applications of plant tissue culture

Besides micropropagation, the applications of plant tissue culture also include the areas of biochemistry, pathology and genetics (Bhojwani and Razdan 1983).

Biochemical applications of plant tissue culture are associated with the production of secondary metabolites by specific tissues or organs during the culture period (Dodds and Roberts 1985). Many pharmaceutical products and other industrial products, such as food flavouring agents and perfumes, are derived from plant products. However, in many cases, the plants may not provide the optimum production of the required compound. The availability of a method for *in vitro* production of these secondary metabolites is therefore an advantage to the industry. However, for large scale industrial production, there is a high cost involved for the production of these secondary metabolites.

A contribution of plant tissue culture to the plant pathology area is the production of pathogen-free plants (Bhojwani and Razdan 1983). For example, virus-free plants have been succesfully recovered from virus infected plants of several species including dahlia, pineapple, ginger, orchid, potato and carnation using meristem tip culture (Bhojwani and Razdan 1983; Gauheret 1985). Production of virus-free plants via meristem tip culture is based on the fact that in infected plants the apical meristems are generally either virus-free or carry a very low concentration of the virus. Therefore, using the meristem tip as an explant will often yield virus-free

plantlets. However, meristem tip culture is sometimes not sufficient for virus eradication. For example, potato virus S (PVS) and potato virus X (PVX) could not be eliminated until the meristem was taken from the heat-treated potato plants (Bhojwani and Razdan 1983).

Another area of plant tissue culture application is genetics. Since the discovery of plant tissue culture, several methods for plant genetic improvement have been developed, including anther and pollen culture, somatic hybridization by protoplast fusion, and *Agrobacterium*-mediated transformation (Gautheret 1985).

The purpose of anther and pollen culture is to produce haploid plants by the induction of embryogenesis from repeated divisions of monoploid spores. The chromosome complement of these haploid plants can then be doubled by colchicine to yield fertile homozygous diploids (Dodds and Roberts 1985).

Plant protoplasts represent a finest single cell system, and therefore offer exciting possibilities for crop improvement. Somatic hybridization through protoplast fusion is initially based on the ability of protoplasts to fuse with each other. This type of hybridization is especially significant in plant improvement programs for plants that have weak sexual reproduction (Bhojwani and Razdan 1983).

Agrobacterium-mediated transformation is the most recent application of plant tissue culture in genetics (For review see the *Agrobacterium* section).

1.5 Tissue culture of ginseng (*Panax* sp)

Tissue culture of both North American (*Panax quinquefolium* L.) and Korean (*Panax ginseng* C.A. Meyer) ginseng was initiated first in 1968 by Butenko and her colleagues. In their work, ginseng callus was established from various explants including leaf, petiole and stem. However, no regeneration was observed. The development of organized structures from these calli was limited to the roots. Although, they did not achieve regeneration, Butenko et al. (1968) did observe that

leaf, petiole and root tissues of ginseng exhibited a high capacity for callus formation and spontaneous somatic embyogenesis during 12-18 months of culture.

A successful regeneration system for Korean ginseng *in vitro* was first established with root explants (Chang and Hsing 1980 a). In this work, globular and heart-shape stage embryos were initiated from root-derived calli after 8 months of incubation on Murashige and Skoog (MS) basal medium supplemented with 2,4-D (4.5 μM). These embryos were then transferred to half-strength MS or B5 medium with GA3 at 2.9 μM and 6-benzylamino purine (BAP) at 4.4 μM for germination. However, these shoots failed to produce roots. Furthermore, if the embryos were isolated from callus and subcultured onto half-strength BM medium with BA (4.4 μM) and GA3 (1.4 μM) or B5 medium with BA (4.4. μM) and GA3 (2.9 μM), these embryos were able to flower. Approximately 90% of the pollen grains proved to be fertile (Chang and Hsing 1980 b).

Since 1980, numerous research projects have been conducted to establish a regeneration system for Korean ginseng using various explant sources including root, young flower buds, seeds and protoplasts (Table 1). In all the experiments, 2,4-D, and kinetin alone or in combination, were the most common growth regulators used for both callus and somatic embryo development from different types of explants, including root, young flower buds, seeds and protoplasts, whereas BAP and GA_3 were the most common growth regulators used for plantlet establishment (Table 1).

Callus culture of North American ginseng was first reported in 1968. However, successful regeneration of plantlets was not reported until 1990 (Wang 1990). In this work, the regeneration of plantlets from root explants was reported. Optimal callus growth was observed on MS medium supplemented with dicamba (9.0 μM). For somatic embryo initiation, embryogenic calli were transferred to MS medium with naphthaleneacetic acid (NAA) (2.15 μM) and 2,4-D (4.52 μM). Plantlets were then

regenerated on MS medium with NAA (0.54 μM) and IBA (2.46 μM), with a 30% frequency of regeneration. Unfortunately, these plantlets were difficult to maintain and no roots were formed. The time required for the entire process of plantlet establishment using root explants was not stated.

Table 1. Reports of tissue culture and plantlet regeneration in Oriental (*Panax ginseng* C.A. Meyer) and North American ginseng (*Panax quinquefolium* L.) since 1980.

Explant Source	Basal Medium	Growth Regulators	Response	References
a) *P. ginseng* C.A.Meyer				
Root	MS	2,4-D (4.52 µM)	Embryoids	Chang and Hsing (1980 a)
	1/2 MS or B5	GA_3 (2.9 µM) + BAP (4.4 µM)	Shoots	
Root	MS	2,4-D (4.52 µM) + Kinetin (0.46 µM)	Callus	Furuya et al. (1986)
	MS	Kinetin (4.65 µM)	Shoots	
Young flower bud	MS	2,4-D (4.5 µM)	Embryoids	Shoyama et al. (1987)
	MS	GA_3 (1.4 µM) + BAP (11.1 µM)	Shoots	
Seed	MS	2,4-D (4.52 µM) + Kinetin (0.046 µM)	Plantlets; in vitro flowering	Lee et al. (1990)
Protoplast	MS	2,4-D (4.52 µM) + Kinetin (0.046 µM)	Embryoids	Arya et al. (1991)
	MS	BAP (4.4 µM) + GA_3 (2.9 µM)	Plantlet	
Root	MS	2,4-D (2.26 µM) + BAP (0.44 µM)	Callus	Jiu (1992)
	MS	2,4-D (4.52 µM)	Embryoids	
Seed	MS	2,4-D (4.52 µM)	Embryoids	Arya et al. (1993)

Table 1 (Cont.)

Explant Source	Basal Medium	Growth Regulators	Response	References
b) *P. quinquefolium* L.				
Root	MS	2,4-D (9.0 μM) + Kinetin (5.0 μM)	Callus	Wang (1990)
	MS	Dicamba (9.0 μM)	Embryogenic calli	
	MS	NAA (0.54 μM) + IBA (2.46 μM)	Plantlets	

1.6 Transformation of plants by *Agrobacterium tumefaciens*

Agrobacterium, the causative agent of plant diseases called crown gall, is a member of the family Rhizobiaceae and closely related to members of the genus *Rhizobium*. The major characteristic of crown gall disease is the formation of tumours at the wound sites of infected plants. This tumour formation is due to the natural capacity of *Agrobacterium* to introduce a segment of DNA from its large tumour-inducing plasmid (Ti plasmid) into plant cells. (Melchers and Hooykaas 1987). This segment of the Ti plasmid is called transferred DNA (T-DNA) and contains genes that encode enzymes involved in the production of an auxin, a cytokinin and specific metabolites called opines. As a result of the introduction of this T-DNA segment into the plant nuclear DNA, the transformed plant cells produced tumorous tissues and were able to grow on growth regulator-free medium (Klee and Rogers 1989; Zambryski 1992).

The discovery of this natural gene transfer system by *Agrobacterium* raised the possibility that it could be adapted for the purpose of introducing desirable genes into crop plants. There are several factors that allowed *Agrobacterium* to be used as a vector: (a) the genes present in T-DNA are not essential for the transfer of the T-DNA into the plant nuclear DNA, (b) the T-DNA borders are acting in *cis* for the T-DNA transfer and any DNA inserted between the T-DNA borders, whether the borders are natural or synthetic, will be transferred into the plant cell, (c) the virulence (*vir*) genes outside the T-DNA on the Ti plasmid which are responsible for mediating the T-DNA transfer can act both in *cis* when the T-DNA and the *vir* genes are on the same plasmid, or in *trans* when the T-DNA and the *vir* genes are on separate plasmids, and (d) the foreign DNA integrated into the plant genome is stably inherited in a Mendelian manner (Walden 1988; Zambryski 1992).

1.6.1 *Agrobacterium tumefaciens* as a plant transformation vector

As previously described, the ability of *Agrobacterium* to transform plants is due to the presence of its Ti plasmid, a circular double-stranded DNA molecule of about 200 kb. The Ti plasmid consists of four important regions: (1) T-DNA (2) virulence (3) plasmid conjugation and (4) origin of replication. Among these four regions, only T-DNA and the virulence region are directly involved in the transformation process (Walden 1988).

The T-DNA consists of two major regions: the hormone synthesizing genes and the opine synthesizing genes, and is defined and delimited by two 25-bp direct repeats at the ends called the T-DNA borders (Barker et al. 1983). Any DNA between these borders will be transferred into the plant cell. Studies suggested that manipulations of the left border have minimal effect on the transformation event, while the right border is critical (Wang et al. 1984).

The *Vir* region is another part of Ti plasmid that contains genes involved in T-DNA excision and transfer. This region is about 35 kb in size and consists of six operons, referred to as *vir* A, B, C, D, E and G (Klee et al. 1987). The expression of these *vir* gene occurs at very low levels under normal conditions, and is induced by phenolic compounds produced by plants (Stachel and Zambryski 1986). The function of these *vir* genes is now well established. *Vir* A and *vir* G are involved in the regulation of the other *vir* regions. The *vir* A product is a membrane-associated protein and is responsible for recognizing and responding to the presence of phenolic compounds in plant exudates, such as acetosyringone (Winans et al. 1989). This information is then transduced to the *vir* G. *Vir* G subsequently acts as a transcriptional activator of itself and the other *vir* genes. The products of *vir* C and *vir* D are responsible for generating and processing the T-DNA copy, whereas *vir* B and *vir* E products are involved in the formation of the structural components that facilitate the T-DNA movement (Klee and Rogers 1989; Zambryski 1992).

Plant transformation vectors based on the *Agrobacterium* system are divided into two systems: cointegrative and binary systems (Walden 1988; Draper and Scott, 1991). In cointegrating vectors, the vector plasmid is integrated into the T-DNA of a resident Ti plasmid. Therefore, a region of homology between the vector plasmid and the Ti plasmid is very important. As a result, this requirement for a homologous segment of DNA limits the vector to one or a few specific Ti plasmids (Klee and Rogers 1989)

The binary system consists of a Ti-plasmid which provides the *vir* functions in *trans* but contains no T-DNA and a separate vector containing the genetically engineered T-DNA. The advantage of the binary vectors over the cointegrative system is that there is no requirement of homology between the vector plasmid and the Ti plasmid (Klee et al. 1987).

1.6.2 Factors that affect *Agrobacterium*-mediated transformation

Explants such as leaves, stems, hypocotyls and roots are the most common types of materials being used for plant genetic transformation studies with *Agrobacterium* (Walden 1988). The efficiency of transformation and transgenic plant production depend on several factors, such as the availability of optimal protocols for inoculation and subsequently for selection and regeneration of transformed cells (Draper et al. 1988). For example, transformation efficiency was reported to be influenced by the type and age of the tissue (Armstead and Webb 1987; Chabaud et al. 1988), the size of explants (Sarmento et al. 1992), the length of the coculture period (Chabaud et al. 1988; Fillati et al. 1987; Sarmento et al. 1992) and the duration of growth prior to inoculation (McHughen et al. 1989; Sangwan et al. 1990). Different *Agrobacterium* strains and culture conditions are also a major factor for achieving successful transformation (Fillati et al. 1987; Hobbs et al. 1989; Byrne et al. 1987).

1.6.3 Confirmation of transformation

Confirmation of transformed tissues or transgenic plants can be performed through the following approaches: phenotypic assay, enzymatic assay, genomic DNA assay, Western blot analysis, and analysis of progeny (Walden, 1988). The phenotypic assay is based on the ability of the transformed cells or tissues to grow under dominant selection, for example, in the prescence of kanamycin or hygromycin, whereas, enzymatic assay is used if the genetic marker is an enzyme, e.g. Chloramphenicol Acetyl Transferase (CAT) (Walden 1988). Confirmation of the integration of the foreign DNA into the plant genome can be performed with Southern blot and Polymerase Chain Reaction (PCR) (Scott et al. 1988). The expression of the foreign DNA to produce protein in the trangenic plant can be confirmed with Western blot analysis (Neuhaus et al. 1991).

1.7 The use of chitinases in plant genetic engineering

1.7.1 Role of chitinases in plant defence against pathogens

In nature, plants have evolved a broad range of mechanisms to defend themselves against invasion by pathogenic microorganisms, which involve the activation of host defence genes. The activation of these host defence genes will create physical and biochemical changes which enable the plant to become more resistant to the invading microorganisms. The biosynthesis and accumulation of several compounds, such as phytoalexins, inhibitors of pathogen enzymes, and lytic enzymes, are associated with this development of plant resistance against pathogens. These enzymes are called pathogenesis-related (PR) proteins (Broglie and Broglie 1993). Currently, there are at least five different groups of PR-proteins recognized: PR-1, PR-2 (such as β-1,3-glucanase), PR-3 (such as chitinases), and

PR-4 and PR-5 (Stinzi et al. 1993). To date, the most frequently described group of PR-proteins are PR-2: (β-1,3 glucanase) and PR-3: (chitinases).

Chitinases are lytic enzymes found in most higher plants, and are defined as enzymes which catalyze the hydrolysis of chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (Collinge et al. 1993). Chitin is a common constituent of insect exoskeletons, shells of crustaceans and fungal cell walls. Chitinase activity in plants is generally elevated by fungal pathogen infection. Several mechanisms of chitinase activity against fungal pathogens have been proposed, such as degradation of fungal cell walls, inhibition of fungal growth, and release of elicitors that may induce other defence mechanisms in plants (Boller 1988). The proposal of chitinase importance in plant defence mechanism is based on the following evidence: (1) purified chitinases are able to inhibit fungal growth and cause the hyphal tips of fungi to lyse *in vitro* (Schlumbaum et al. 1986; Sela Buurlage et al. 1993); (2) in healthy plants, chitinases are usually expressed at a very low level and treatment with the phytohormone ethylene, oligosaccaharide elicitors, or infection by fungal pathogens will increase the level of chitinases (Metraux et al. 1986; Punja and Zhang 1993) and (3) chitin is an important part of fungal cell walls and is not found in plants (Metraux 1986).

1.7.2 Plant chitinases

Plant chitinases have molecular weights ranging from 25 to 36 kD, are usually stable at temperatures of up to 50 ^{o}C and may be either acidic or basic (Collinge et al. 1993). Based on the final products after hydrolytic reactions, chitinases can be classified as endochitinases or exochitinases. To date, endochitinases are the types of chitinase that have been most extensively studied. They randomly hydrolyze internal β-1,4-linkages of chitin to release oligomers of β-1,4-N-acetylglucosamine of

various lengths, whereas exochitinase hydrolyzes chitin from its reducing terminal end (Collinge et al. 1993; Tronsmo and Harman 1993).

1.7.3 Modification of chitinase gene expression in transgenic plants

As mentioned above, various *in vitro* studies have provided evidence for the antifungal properties of these enzymes. However, reports of constitutive expression of these chitinase genes in transgenic plants have shown mixed results in response to fungal infection (Table 2).

Table 2. Constitutive expression of chitinase genes in transgenic plants in relation to resistance to fungal pathogens.

Chitinase source	Expression in transgenic plants
Class I Chitinase (tobacco)	No resistance to *Cercospora nicotianae* observed in transgenic *Nicotiana sylvestris*
Class III Chitinase (sugar beet)	No increase in resistance to *C. nicotianae* in transgenic *Nicotiana benthamiana*
Class I Chitinase ; (bean)	Increased resistance to *Rhizoctonia solani* observed in transgenic tobacco and canola
Class I Chitinase (tobacco)	No resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 1 obseved in transgenic tomatoes

Sources : Neuhaus et al. 1991; Nielsen et al. 1993; Broglie and Broglie, 1993; Van den Elzen et al. 1993.

1.8 Objectives of Research

To date, regeneration of North American ginseng (*P. quinquefolium* L.) *in vitro* have only been established using root explant. A primary problem faced by ginseng growers is plant disease. Due to these disease problems, ginseng production can be reduced up to 60%. Breeding programs of North American ginseng using conventional method will face obstacles such as the long reproductive cycle of the plant, interspecific cross breeding, and 18-20 months of seed dormancy. Elevating the chitinase levels by introducing foreign chitinase genes into plants has been proposed as one possible way to improve plant resistance to fungal pathogens.

The objectives of this research are:

1. To develop methods for efficient and reliable regeneration of North American ginseng through somatic embryogenesis using root, leaf and epicotyl explants.

2. To develop procedures to introduce a chitinase gene into North American ginseng using *Agrobacterium*-mediated transformation.

CHAPTER II

Somatic Embryogenesis and Plantlet Regeneration in North American ginseng (*Panax quinquefolium* L.)

2.1 Introduction

Ginseng (*Panax sp.*) is a slow-growing perennial herbacious plant which is used as a source of vitalizing and stimulating agents (Proctor and Bailey 1987). There are several known *Panax* species, including *P. quinquefolium* L. (North American ginseng) and *P. ginseng* C.A. Meyer (Oriental/Korean ginseng), which represent the predominant species grown in North America and Asia, respectively.

The primary method of ginseng propagation is through seeding. However, there are several problems associated with this method, including the long reproductive and production cycle (3-4 years) before the plant produces seeds, an 18-20 month seed dormancy period and seed germination rates of 50-60% (Proctor and Bailey 1987). For these reasons, plant improvement using conventional breeding approaches for ginseng would be very slow and difficult to conduct. Tissue culture methods could provide an alternative approach to overcome these problems. With tissue culture, propagation of genotypically superior lines could be achieved more rapidly, and variability associated with seed propagation could be reduced. Furthermore, the ability to regenerate plants *in vitro* could also provide opportunities for plant transformation and the development of transgenic plants.

To date, several regeneration systems using various explant types, such as root, seed, young flower bud and protoplasts, have been developed for Korean ginseng (Chang and Hsing 1980a; Furuya et al. 1986; Shoyama et al. 1986; Lee et al. 1990; Arya et al. 1991; Jiu et al. 1992; Arya et al. 1993; Table 1). For American

ginseng, however, a regeneration system using tissue culture has only been achieved using root explants (Wang 1990).

The objectives of this study were to develop a method for efficient regeneration of plantlets of North American ginseng through somatic embryogenesis by comparing root, leaf and epicotyl explants and to determine the optimal conditions for plantlet development.

2.2 Materials and Methods

2.2.1 Tissue culture media and culture conditions.

The basal medium used in this study was MS (Murashige and Skoog, 1962) supplemented with myo-inositol (100 mg/l), thiamine-HCl (0.1 mg/l), nicotinic acid (0.5 mg/l), pyridoxine-HCl (0.5 mg/l), glycine (2 mg/l), sucrose (30 g/l) and tissue culture agar (9 g/l). All chemicals used were tissue culture grade (Sigma Chemical Co., St. Louis, MO). The pH was adjusted to 5.8 with 1 N NaOH or HCl prior to autoclaving at 15 psi for 15 min. For experiments on callus induction and somatic embryo development from different explant sources, the dishes were incubated in the dark at 22-26 ^{o}C for periods of 3 to 9 months, depending on the type of explants. For somatic embryo germination, cultures were incubated either at 4 ^{o}C or at ambient temperatures of 22 to 26 ^{o}C under low light (intensity of 7.0 $\mu mol.m^{-2}.s^{-1}$) provided by cool-white fluorescent lamps with a 16 h/day photoperiod . In all treatments, to evaluate the effect of different parameters on embryo formation, 9-10 replicate petri dishes, each with 7 -10 explants, were used. The experiments were repeated at least once. The explant sources evaluated in this study were from root, leaf and epicotyl.

2.2.2 Plant Materials

2.2.2.1 Root explants.

Callus development. Ginseng plants (ranging from 4 to 5 years old) grown under field conditions in Lillooet, B.C. (courtesy of Dr. Eric R. Littley, Chai-Na-Ta Farms), were uprooted and brought back to the laboratory. The taproot was subsequently washed in water before surface sterilization in 70% ethanol for 7 min and in a 40% solution of commercial bleach (Javex, 6.25% sodium hypochlorite) for 10 min, followed by three to five rinses in sterile distilled water. The epidermal tissues were discarded and pith sections (0.4-0.6 $cm^{2)}$ were removed from the root and plated in petri dishes (100x15 mm) containing approximately 25 ml of MS medium. The combinations of growth regulators that were evaluated for callus induction (CD 1-CD 25) are shown in Table 3. A few petri dishes were incubated under light (intensity of 30 $\mu mol.m^{-2}.s^{-1)}$ and the remainder under complete darkness. These experiments showed that optimal growth of root-derived callus occured on media CD 3, CD 7, CD 8, and CD 15 in complete darkness. Further experiments were therefore established to compare the growth of root-derived callus on each of these four growth regulator combinations. The diameter of calluses on dishes incubated in the dark was measured at 2-week intervals for up to 3 months. For each treatment, a total of 72 calluses were measured.

Table 3. Growth regulator combinations (in μM) evaluated for promotion of callus development.

Medium*	Dicamba	Kinetin	BAP	2,4-D	NAA
CD1	4.5	-	-	-	-
CD2	6.8	-	-	-	-
CD3	9.0	-	-	-	-
CD4	11.3	-	-	-	-
CD5	13.5	-	-	-	-
CD6	4.5	5.0	-	-	-
CD7	4.5	10.0	-	-	-
CD8	9.0	5.0	-	-	-
CD9	13.5	5.0	-	-	-
CD10	-	2.3	2.2	2.2	-
CD11	-	4.6	4.4	4.4	-
CD12	-	6.9	6.6	6.6	-
CD13	-	9.2	8.8	8.8	-
CD14	-	5.0	-	4.5	-
CD15	-	5.0	-	9.0	-
CD16	-	-	-	4.5	-
CD17	-	-	-	9.0	-
CD18	-	5.0	-	-	5.0
CD19	-	5.0	-	-	10.0
CD20	-	5.0	-	-	15.0
CD21	-	-	-	9.0	5.0
CD22	-	-	-	9.0	10.0
CD23	-	-	-	9.0	15.0
CD24	-	-	-	-	5.0
CD25	-	-	-	-	10.0
CD26	-	2.5	-	-	5.0
CD27	-	5.0	-	-	7.5
CD28	-	7.5	-	-	10.0
CD29	-	10.0	-	-	12.5

* MS basal medium supplemented wih the indicated combinations of growth regulatos.

Somatic embryo development. After 3-4 months of incubation, embryogenic calluses which developed on medium CD 7 and CD 8 (Table 3) were transferred to a somatic embryo development (SED) medium. The combinations of growth regulators tested to promote somatic embryo development are listed in Table 4. All cultures were incubated in the dark. In these experiments, somatic embryo development occurred on media SED 1, SED 2, SED 3, and SED 6. In additional experiments, embryogenic calluses developing on different CD media were transferred as follows: from CD 8 to SED 1 and SED 3, from CD 9 to SED 2, from CD 7 to SED 6, and from CD 15 to SED 3 (Table 2 and Table 3). All cultures were incubated in the dark and the percentage of calluses with globular somatic embryos (the number of calluses producing embryos/the total number of calluses) was determined 3-4 months after transferring to SED media.

The effects of phenylboronic acid (PBOA) and silver nitrate ($AgNO_3$) in promoting somatic embryo formation were also evaluated. Calluses developing on medium CD 8, after 3 months of growth were recut into 0.6-0.8 cm^2 pieces and replated onto the same medium, and supplemented with 0, 2, 5.and 10 μM of PBOA. Each treatment had 10 plates, each with eight calluses. Somatic embryo formation was recorded 3 and 6 months after transfer. Root-derived calluses developing on medium CD 9, were also recut into 0.6-0.8 cm^2 pieces and replated onto the same medium, and supplemented with 5, 10, 15 or 20 mg/l of $AgNO_3$. All cultures were incubated in the dark. The percentage of somatic embryo formation was recorded at monthly intervals up to 10 months after transfer to SED medium.

Table 4. Growth regulator combinations (in μM) evaluated for promotion of somatic embryo development (SED) by root explants.

Medium*	Dicamba	Kinetin	NAA	2,4-D	2-ip
SED1(=CD1)	4.5	-	-	-	-
SED2(=CD2)	6.8	-	-	-	-
SED3(=CD3)	9.0	-	-	-	-
SED4(=CD6)	4.5	5.0	-	-	-
SED5(=CD7)	4.5	10.0	-	-	-
SED6(=CD8)	9.0	5.0	-	-	-
SED7(=CD9)	13.5	5.0	-	-	-
SED8	-	-	1.25	2.25	-
SED9	-	-	2.5	4.5	-
SED10	-	-	5.4	-	4.9
SED11	-	-	5.4	-	9.8
SED12	-	-	10.8	-	4.9
SED13	-	-	10.8	-	9.8
SED14	-	-	16.2	-	4.9
SED15	-	-	16.2	-	9.8

* MS basal medium supplemented with the indicated combinations of growth regulators.

2.2.2.2 Leaf explants from lab-grown seedlings.

Callus development and somatic embryo formation.

Dehusked seeds of ginseng which had been previously stratified over a 9-12 month period with a fluctuating cool:warm:cool temperature regime (courtesy of Dr. Eric R. Littley, Chai-Na-Ta Farms, Kamloops, B.C.) were immersed in 70% ethanol for 5 min and then a 20% solution of Javex for 10 min and rinsed three-five times in sterile water. The seeds were plated on MS medium supplemented with 6-benzylaminopurine (BA) at 4.4 μM and gibberellic acid (GA_3) at 2.9 μM. All dishes were incubated at 4°C under low light (intensity of 7.0 $\mu mol.m^{-2}.s^{-1}$) with a 16h/day photoperiod. When the seeds began to germinate, they were transferred to ambient temperatures of 22 to 26 °C with the same light conditions. The cultures were transferred at monthly intervals to the same medium until the cotyledons emerged and seedlings were obtained (5-8 months from initial plating).

Leaf segments (0.2-0.4 cm^2) from these *in vitro* -derived seedlings were plated onto media CD 1-CD 5, CD 14-CD 17, CD 21-CD 23, and CD 26-CD 29 (Table 3). For each treatment, there were 10 petri dishes, each containing seven leaf segments. A few dishes were placed under light and the remainder in the dark. These experiments showed that CD 22 was suitable for somatic embryo development. Therefore, the growth rate of seedling leaf-derived callus and the percentage of calluses with somatic embryos were measured on this medium over a 3-month period.

2.2.2.3 Epicotyl explants from lab-grown seedlings.

Callus development and somatic embryo formation.

Epicotyl segments (0.2-0.3 cm long) obtained from lab-grown seedlings were plated onto media CD 1-CD 9, and CD 21-CD 23 (Table 3). For each treatment, there

were 10 petri dishes, each with seven explants. The dishes were incubated in the dark for a 6 to 9 month period and the percentage of somatic embryo formation was recorded at monthly intervals.

2.2.2.4 Mature leaf explants.

Callus development and somatic embryo formation.

Mature leaves from 3-, 4- and 5-year old field grown plants from Lillooet, B.C. (courtesy of Dr. Eric R. Littley, Chai-Na-Ta Farms) were dipped in 70% ethanol for 25 sec and then in a 10% solution of Javex for 10 min and subsequently rinsed three to five times with sterile distilled water. Leaf segments (0.2-0.4 cm^2) were plated onto MS medium with the various combinations of growth regulators shown in Table 5 and designated as MA media. Mature leaf derived calluses appeared to develop best on medium MA 13, MA 14, MA 15, and MA 16, whereas somatic embryo formation was observed on medium MA 19, MA 20, and MA 21. Therefore, further experiments with MA 13, MA 14, MA 15, MA 16, MA 19, MA 20, MA 21, and MA 22 were repeated. For each treatment, there were 10 petri dishes, each containing seven explants. The growth of calluses was measured every 2 weeks for up to 3 months and the percentage of calluses with somatic embryos was recorded after 3 and 7 months of incubation in the dark for all treatments.

Table 5. Growth regulator combinations (in μM) evaluated for callus and somatic embryo development by mature leaf explants

Medium*	NAA	2-ip	Kinetin	Dicamba	2,4-D
MA 1	5.4	9.8	-	-	-
MA2	10.8	4.9	-	-	-
MA3	16.2	4.9	-	-	-
MA4	16.2	9.8	-	-	-
MA5	2.5	-	4.6	-	-
MA6	5.0	-	4.6	-	-
MA7	7.5	-	5.0	-	-
MA8	10.0	-	7.5	-	-
MA9	5.0	-	2.5	-	-
MA10	2.5	-	9.3	-	-
MA11	5.0	-	9.3	-	-
MA12	12.5	-	9.3	-	-
MA13	-	-	5.0	4.5	-
MA 14	-	-	5.0	9.0	-
MA15	-	-	5.0	13.5	-
MA16	-	-	10.0	4.5	-
MA17	-	-	5.0	-	4.5
MA18	-	-	5.0	-	9.0
MA19	5.0	-	-	-	9.0
MA20	10.0	-	-	-	9.0
MA21	15.0	-	-	-	9.0
MA22	20.0	-	-	-	9.0

* MS basal medium supplemented with the indicated combinations of growth regulators.

2.2.3 Plantlet development.

2.2.3.1 Effect of BA, GA, kinetin, NAA and IBA.

In preliminary experiments, somatic embryos originating from root, leaf or epicotyl explants were placed on MS medium supplemented with BA at 4.4 μM and GA3 at 2.9 μM as suggested by Chang and Hsing (1980a). However, in all experiments, only shoots developed. To promote root formation, the shoots were excised and transferred to MS medium with kinetin and GA3 at 4.6 and 1.9 μM, respectively; NAA/IBA (0.5/2.5 μM); half-strength MS salts + 9.8 μM IBA, or to MS0. For each treatment, 10 shoots were incubated either at 4 ^{o}C or at ambient temperature of 22-26 ^{o}C with the same light conditions as before. The shoots were examined periodically and the percentage of root formation was recorded at monthly intervals for up to 6 months.

2.2.3.2 The effect of ABA.

The effect of ABA on plantlet development was also examined. Calluses (20-30 mg) from seedling-derived leaf explants with somatic embryo at different stages of deveopment (globular, torpedo and cotyledonary) were precultured in MS medium with NAA/2,4-D (10.0/9.0 μM) supplemented with ABA at 0, 5, 10, 20, 40 μM. Each plate contained four calluses, with five replications per treatment; all dishes were incubated in the dark at ambient temperatures of 22-26 ^{o}C. After 3, 4, 6 and 8 weeks of preculture in the above medium, 20 mature somatic embryos were transferred to MS medium with BA/ GA_3 (4.4/2.9 μM) and incubated at 4 ^{o}C under the same light intensity as before. The number of somatic embryos germinating as well as the percentage of plantlet formation were recorded at 4 week intervals from 4 to 20 weeks.

2.2.3.3 The effect of charcoal.

The effect of activated charcoal (1%) on embryo development was also evaluated in MS medium with BA/GA_3 (4.4/2.9 μM), NAA/2,4-D (10.0/9.0, 5.0/4.5 μM), or with half-strength MS salts. Calluses containing two-four mature somatic embryos derived from seedling leaf explants were placed on the above media using four replicate plates of each treatment. All cultures were incubated at ambient temperatures of 22-26 ^{o}C with low light intensity. Shoot development was rated after various times (3, 5, 7, 14, 21, 28 and 35 days), whereas the number of plantlets (shoots with roots) was rated at 2 week intervals, after 1 to 3 months of culture.

2.2.4 Statistical analysis

Callus growth from different explants as well as percentage of shoot and root development on different media, were analyzed for significant treatment effects at the last observation date. The data from the replications were analyzed using ANOVA and mean separation among treatments was achieved using Tukey HSD at P=0.05.

2.3 Results

2.3.1 Root explants.

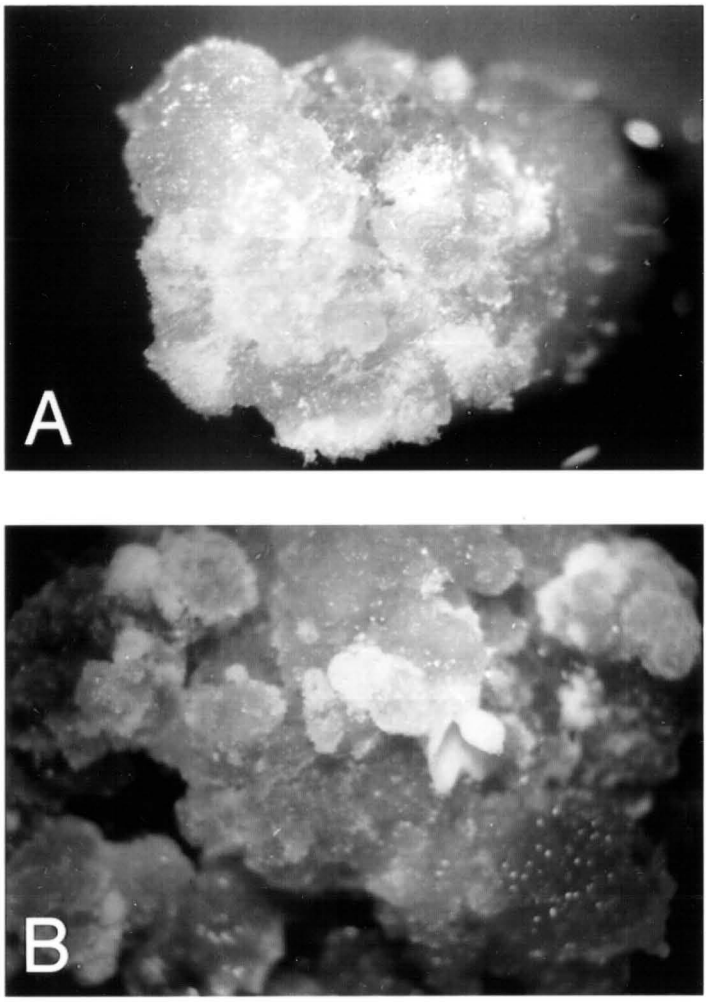
Callus and somatic embryo development.

Within 2-3 weeks of plating, pith sections from ginseng roots began to develop callus of a pale yellow colour and was generally compact in appearence (Fig. 1A). Preliminary experiments showed that rapid callus growth occurred only on media CD 3, CD 7, CD 8, and CD 15 (Table 3), whereas, callus growth on all of the other growth regulators tested was unsatisfactory. Callus growth on the five growth regulator combinations above was therefore measured at 2-week intervals. During the first 6 weeks, there were no significant differences between the treatments with regard to callus growth. After 10 weeks, callus growth was significantly affected by some of the treatments ($P \leq 0.01$), with the largest diameter of 1.72 cm on medium CD 8. The average diameters on media CD 7, CD 3, and CD 15 were 1.54 cm, 1.53 cm and 1.29 cm, repectively, and these were not significantly different from each other (Fig. 2).

During the first 3 months following transfer to callus induction media, no somatic embryos were obtained on media CD 1 - CD 25 (Table 3). Upon transfer from CD 8 to SED 3 medium and incubation for 3 months, 15.6% of the root-derived calluses formed somatic embryos (Fig. 1B), and a total of 26 embryos were obtained. No somatic embryos developed on any of the other growth regulator combinations tested.

The addition of PBOA or $AgNO_3$ was found to have no effect in promoting somatic embryo development at any of the concentrations tested.

Fig. 1. Embryogenic callus and somatic embryo originating from root explants of *Panax quinquefolium* L. **A)** Root-derived embryogenic callus growing on CD 8 medium (MS medium with 9.0 μM dicamba and 5.0 μM kinetin), after 3 months of culture in the dark. **B)** Root-derived somatic embryo after 6 months of culture in the dark on SED 3 medium (MS medium with 9.0 μM dicamba).


A
B

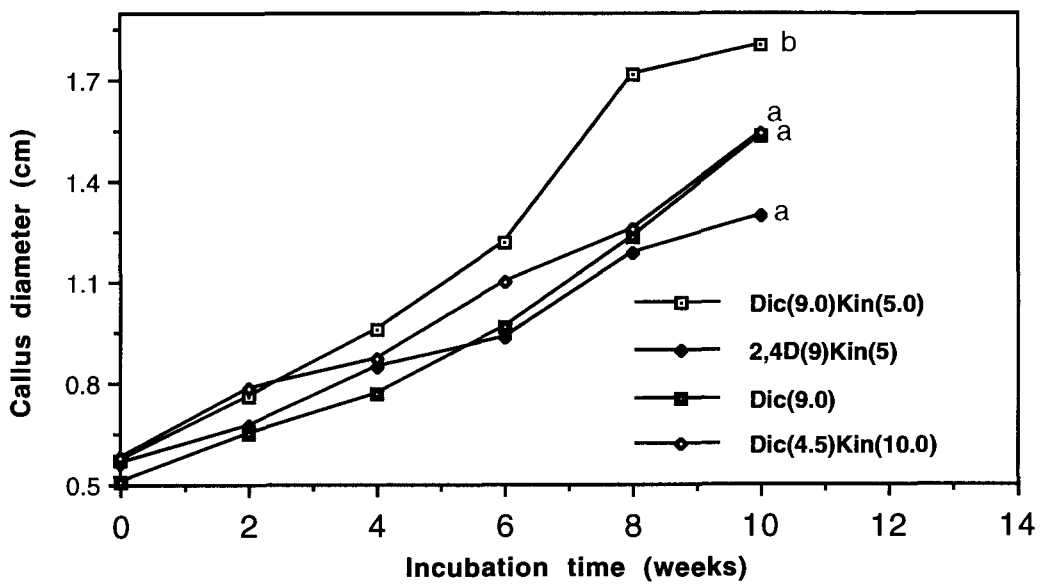


Fig. 2. Growth of calluses derived from root explants of ginseng on MS medium containing different growth regulator combinations: CD8-dicamba 9.0 μM + kinetin 5.0 μM, CD15-2,4-D 9.0 μM + kinetin 5.0 μM, CD3-dicamba 9.0 μM, CD7-dicamba 4.5 μM+kinetin 10,0 μM. Means followed by the same letter are not significantly different (ANOVA, $P \leq 0.01$).

2.3.2 Leaf explants from lab-grown seedlings.

Callus and somatic embryo development.

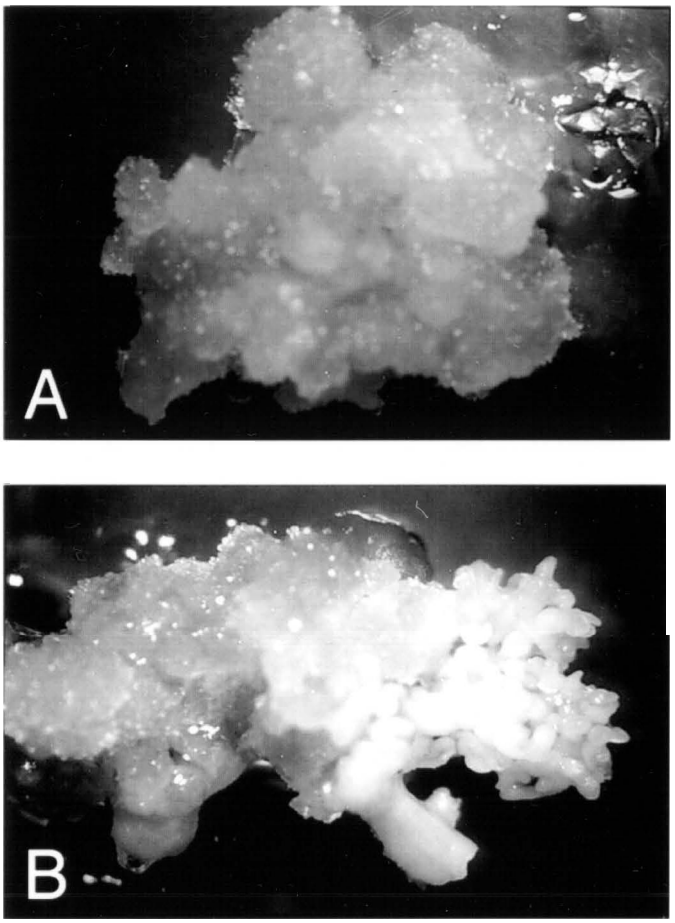
Callus development was observed within 2 weeks, generally at the cut edges of the explant and was soft, watery and friable in appearance (Fig. 3A). Among all of the growth regulators evaluated, the combinations of NAA (10 μM) and 2,4-D (9.0 μM) or CD 22 (MA 20, Table 5) medium gave the highest percentage of somatic embryo formation. The rate of callus growth on this medium is shown in Fig. 4. After 2 months of incubation, the percentage of embryo formation was 30% with a total of 55 embryos were obtained. After 3 months, the frequency of somatic embryo production increased to 40% with a total of 150 embryos obtained (Table 6, Fig. 3B). By transferring the calluses to fresh medium at monthly intervals, the capacity to form somatic embryos in these cultures was retained over 3 years of culture (data not shown).

2.3.3 Epicotyl explants from lab-grown seedlings.

Callus and somatic embryo development.

Epicotyl-derived callus was watery, soft and friable and similar in appearance to that developed from seedling-derived leaf explants. Among all of the growth regulator combinations tested, only calluses on CD 8 medium formed somatic embryos after 9 months of culture (Table 6). The frequency of somatic embryo formation was very low (2%). This experiment was not repeated.

Figure 3. Embryogenic callus and somatic embryo originating from seedling leaf explants. **A)** Seedling leaf-derived embryogenic callus growing on CD 22 (MA 20) medium (MS medium with 10.0 μM NAA plus 9.0 μM 2,4-D). **B)** Seedling leaf-derived somatic embryos 3 months after culture in the same medium.


A
B

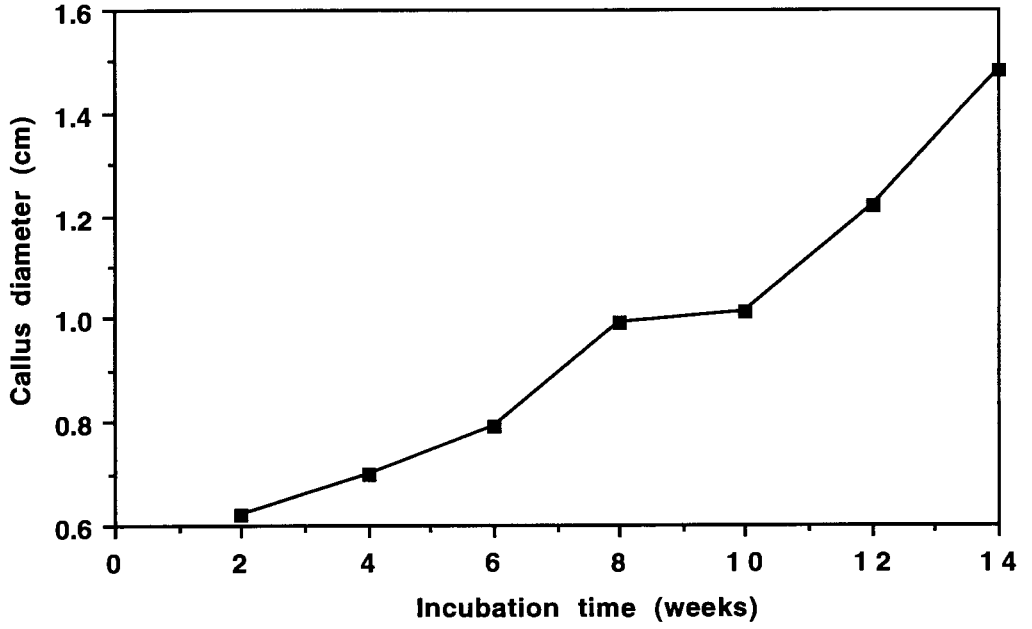


Fig. 4. Growth of calluses derived from lab grown seedling leaves of ginseng on MS medium containing NAA/2,4-D (10.0/9.0 μM).

2.3.4 Mature leaf explants.

Callus and somatic embryo development.

Callus formation first occurred at the edges of the cut leaf explants. When the leaf explants were associated with a midrib or vein, callus growth was better. Among all of the different combinations of growth regulators tested (Table 5), only media MA 13, MA 14, MA 15, MA 16 and MA 19, MA 20, MA 21, MA 22 provided suitable conditions for callus growth of mature leaf explants. After 12 weeks of culture, there were no significant differences ($P=0.350$) in callus growth on the media containing different combinations of NAA and 2,4-D or media MA 19-MA 22, Figure 5, whereas the combination of dicamba and kinetin (media MA13-MA16) significantly affected callus growth ($P \leq 0.01$) with MA 14 (medium containing 9.0 μM dicamba plus 5.0 μM kinetin) producing the largest callus (Figure 6).

After 3 months of incubation, however, none of the calluses from the MA 13, MA 14, MA 15, MA 16, MA 19, MA 20, MA 21, and MA 22 had produced somatic embryos. Somatic embryos developed 7 months after the initial culture, with a frequency of 20% on medium MA 20 and 7.2% on medium MA19 (Table 6).

Table 6. Somatic embryogenesis of North American ginseng (*Panax quinquefolium* L.) from root, epicotyl, mature and seedling leaf explants.

Explant sources	Optimal medium	Somatic embryo formation (%)	Period of incubation (months)
Root	CD 8[a] to SED 3[b]	15.6%	6
Seedling leaf	CD22 (MA 20)[c]	30-40%	2-3
Mature leaf	MA 20[c]	20%	7
Epicotyl	MA 14[d] (9.0/5.0 μM)	2%	9

a MS basal medium with 9.0 μM dicamba and 5.0 μM kinetin.

b MS basal medium with 9.0 μM dicamba.

c MS basal medium with 10.0 μM NAA and 9.0 μM 2,4-D.

d MS basal medium with 9.0 μM dicamba and 5.0 μM kinetin.

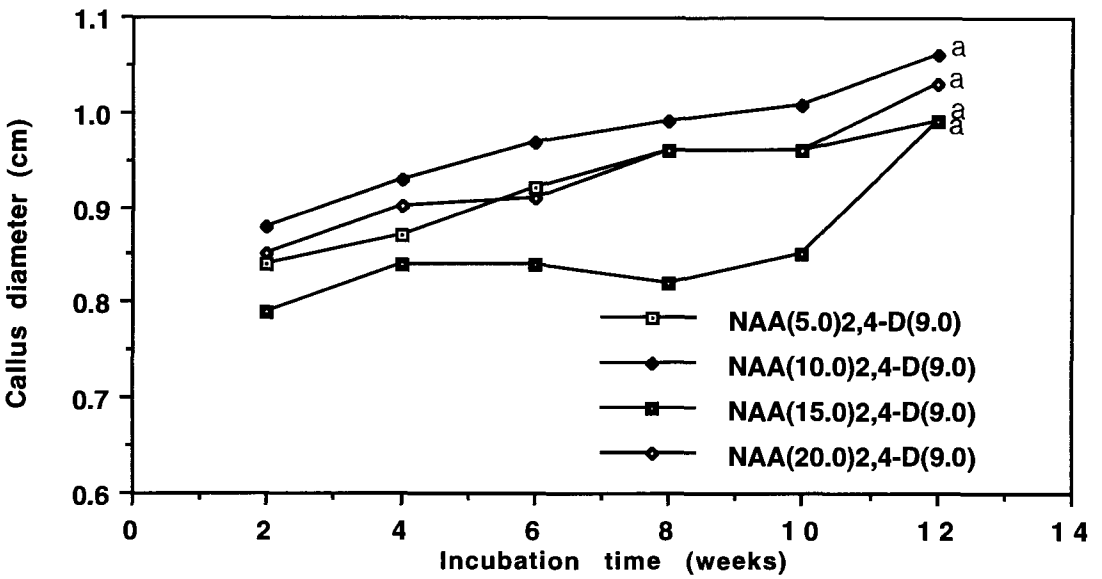


Fig. 5. Growth of calluses derived from 3 year old mature leaf explants of ginseng on MS medium containing 4 different combinations of NAA and 2,4-D: MA19-5.0 μM NAA+ 9.0 μM 2,4-D, MA20-10 μM NAA + 9.0 μM 2,4-D, MA21- 15.0 μM NAA + 9.0 μM 2,4-D, MA22- 20.0 μM NAA + 9.0 μM 2,4-D. Means followed by the same letter are not significantly different (ANOVA, P=0.350).

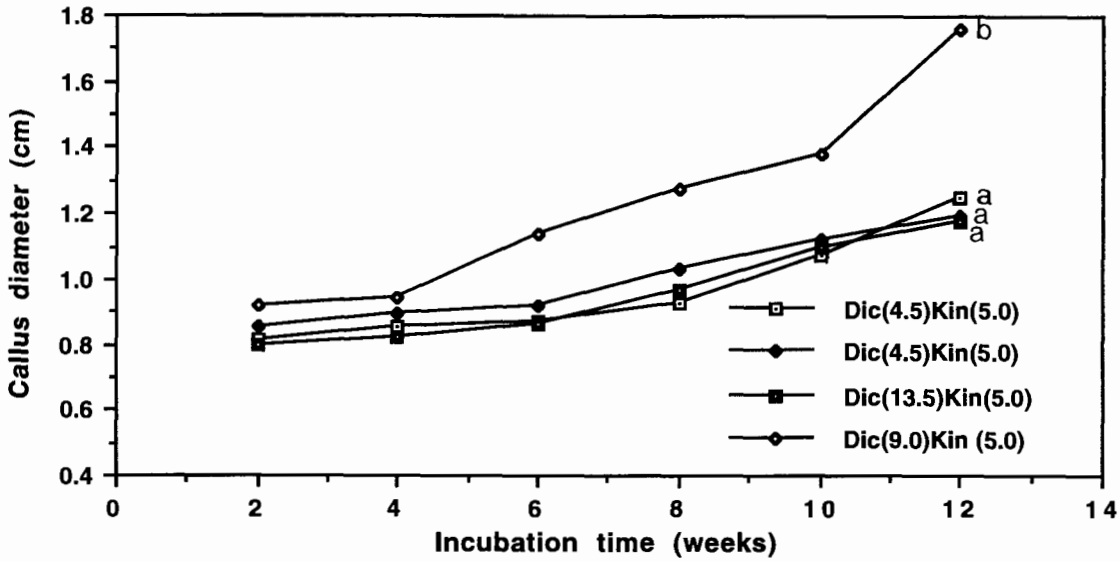


Fig. 6. Growth of calluses from 3 year old mature leaf explants of ginseng on MS medium containing 4 different combinations of dicamba and kinetin: MA13- 4.5 μM dicamba + 5.0 μM kinetin, MA14- 9.0 μM dicamba + 5.0 μM kinetin, MA15- 13.5 μM dicamba + 5.0 μM kinetin, MA16-4.5 μM dicamba + 10.0 μM kinetin. Means followed by the same letter are not significantly different (ANOVA, $P \leq 0.01$).

2.3.5 Effect of BA, GA, kinetin, NAA, and IBA

Shoot development was obtained with BA/GA_3 (4.4/2.9 μM), but these shoots did not produce roots on any of the growth regulators tested (data not shown). The combination of NAA/IBA at 0.5/2.5 μM, kinetin/GA3 at 4.6/1.9 μM, 1/2 strength MS salts plus 9.8 μM IBA or MS0 medium, were found to have no effect in promoting roots from shoots derived from BA/GA_3 (4.4/2.9 μM).

2.3.6 The effect of ABA

No germination was observed from somatic embryos precultured in medium supplemented with different levels of ABA. Different temperature conditions (ambient temperature of 22-26 ^{o}C vs 4 ^{o}C) also had no effect on germination of somatic embryos plated onto media with different concentrations of ABA.

2.3.7 The effect of charcoal

The earliest shoot development was observed with medium supplemented with BA/GA_3 (4.4/2.9 μM) and activated charcoal (1%). After 5 days of incubation, 11.7% of the somatic embryos developed shoots (Table 7). Shoot development frequencies of 6.7% and 23.3% were observed 7 days after culture on medium with NAA/2,4-D (10.0/9.0 μM) and 1/2 MS respectively, whereas shoot development on medium with NAA/2,4-D (5.0/4.5 μM) was observed 14 days after culture (6.7%) (Table 7). After 35 days of culture, the number of shoots formed from embryos was significantly affected by the supplement of different growth regulators with activated charcoal (1%) ($P=0.05$). The highest percentage of shoot development (70%) was achieved on medium half-strength MS medium with charcoal (1%). The combination of NAA/2,4-D (10.0/9.0 μM), BA/GA_3 (4.4/2.9 μM), and NAA/2,4-D (5.0/4.5 μM) showed 36.7%, 25%, and 25% of shoot development, respectively (Table 7). There were no significant differences among these three treatments.

The number of plantlets (germinating somatic embryos with shoot and root) was significantly affected by the different types of growth regulators supplemented with activated charcoal (1%) (P=0.018). After 12 weeks of incubation, the highest frequency of plantlet establishment was 90% on media with NAA/2,4-D (5.0/4.5 μM) (Figure 7A, 7B), followed by NAA/2,4-D (10.0/9.0 μM), BAP/GA_3 (4.4/2.9 μM) and half-strength MS salts, with 62.5%, 52.5% and 41.7%, respectively (Table 8). On medium with NAA/2,4-D (5.0/4.5 μM), the somatic embryos also underwent a process of repetitive embryogenesis. These new somatic embryos directlly formed both shoots and roots axes on this medium.

Table 7. Shoot development of *Panax quinquefolium* L.on different combinations of growth regulators supplemented with activated charcoal.

Medium	Shoot development (%) at various times (days)[u]						
	3	5	7	14	21	28	35[v]
BAP/GA+C[w]	--	11.7	16.7	18.4	21.7	25.0	25.0 **a**
NAA/2,4-D+C[x]	--	--	6.7	18.4	21.7	31.2	36.7 **a**
NAA/2,4-D+C[y]	--	--	--	6.7	13.3	18.4	25.0 **a**
0.5MS+C[z]	--	--	23.3	60	66.7	66.7	70.0 **b**

- [u] Calluses from seedling leaf explants containing mature somatic embryos were used as the starting material.
- [v] Statistical analysis for significant treatment effects was done at day 35. Values in the same column followed by the same letter are not significantly different (Tukey HSD, P=0.05).
- [w] BAP/GA_3 (4.4/2.9 µM) with activated charcoal (1%).
- [x] NAA/2,4-D (10.0/9.0 µM) with activated charcoal (1%).
- [y] NAA/2,4-D (5.0/4.5 µM) with activated charcoal (1%).
- [z] half-strength MS salts with activated charcoal (1%).

Table 8. Plantlet development of *Panax quinquefolium* L. on different combinations of growth regulators supplemented with activated charcoal (1%)

Medium	Plantlet development (%) at various times (weeks)[u]				
	4	6	8	10	12
BAP/GA+C[w]	0	12.5	12.5	37.5	52.5 **a**
NAA/2,4-D+C[x]	22.5	30.0	30.0	50.0	62.5 **a**
NAA/2,4-D+C[y]	6.7	58.3	63.3	76.7	90 **b**
0.5 MS+C[z]	16.7	16.7	31.7	39.5	41.7 **a**

- [u] Calluses from seedling leaf explants containing mature somatic embryos were used as the starting material.

- [v] Statistical analysis for significant treatment effects was done at week 12. Values in the same column followed by the same letter are not significantly different (Tukey HSD, P=0.05).

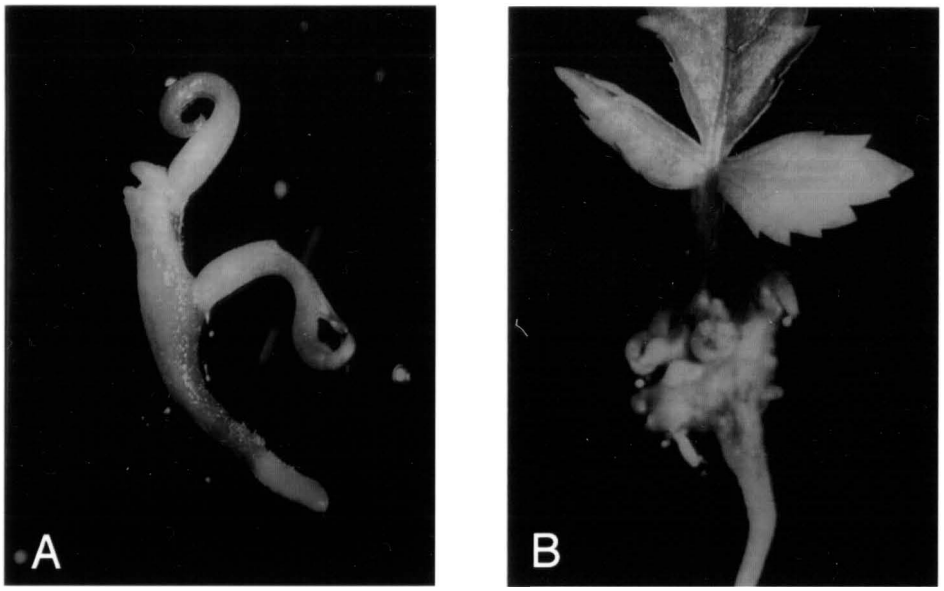
- [w] BAP/GA_3 (4.4/2.9 μM) with activated charcoal (1%).

- [x] NAA/2,4-D (10.0/9.0 μM) with activated charcoal (1%).

- [y] NAA/2,4-D (5.0/4.5 μM) with activated charcoal (1%).

- [z] half-strength MS salts with activated charcoal (1%).

Figure 7. Plantlet development of *Panax quinquefolium* L. on MS medium with NAA/2,4-D (5.0/4.5 μM) and activated charcoal (1%). **A)** Germinating somatic embryo with shoot and root. **B)** Further development of plantlet after 12 weeks of culture on the same medium.


A
B

2.4 Discussion

The results from this study have shown that the development of plantlets of *P. quinquefolium* L. through somatic embryogenesis is influenced by several factors, such as source of explants, age of explants, and type of growth regulators supplemented to the basal medium. The shortest period for somatic embryogenesis was 2 months using seedling-derived leaf explants plated onto medium MA 20, followed by root, mature leaf and epicotyl explants, each plated on medium SED 3 (Table 4), medium MA 20 (Table 5), and medium CD 8 (SED 6) (Table 3 and Table 4), which required 6, 7 and 9 months, respectively (Table 6). Among all of the explants evaluated in this study, the highest frequency of somatic embryo formation was observed with seedling leaf explants (40%) within a 3 month period (Table 6). For both root and epicotyl explants, dicamba with kinetin or dicamba alone were sufficient in promoting both callus growth and somatic embryo development. In contrast, for leaf explants, dicamba with kinetin were optimal solely for callus growth, whereas NAA with 2,4-D were suitable for somatic embryo development. This observation is consistent with the hypothesis by Zimmerman (1993), who stated that the role of exogenous auxin in somatic embryo development depends on the nature of the explants used in the experiment.

This study also demonstrated that different ages of leaf explants, i.e. juvenile (seedling) and mature leaf, showed different responses to the growth regulators supplemented to the medium. Leaf explants derived from juvenile (seedling) plants plated onto medium CD 22 (MA 20) (Table 3, Table 5) were able to produce somatic embryos within a 2 month period, with 30% somatic embryo formation, whereas, mature leaf explants plated onto the same medium did not produce somatic embryos until 7 months after culture, with 20% somatic embryo formation (Table 6). These results suggest that the maturity of leaf explants has a negative influence on somatic embryogenesis in *P. quinquefolium* L. This is consistent with the suggestion by

Zimmerman (1993), who proposed that the highest embryogenic potential of culture explants or cells was within the first year of development.

The earlier work on *P. quinquefolium* L. with root explants (Wang 1990) demonstrated that somatic embryogenesis can be divided into two phases: initiation, and maturation of the embryos. In the present study using leaf explants, both initiation and maturation of somatic embryos were achieved on the same medium. The combined of initiation and maturation of somatic embryos into a one-step process has also been reported for other plants, such as peanut (Hazra et al. 1989) and chilli pepper (Harini and Sita 1993).

With regard to plantlet recovery, the combination of BAP/GA_3 at 4.4/2.9 μM, as suggested by Chang and Hsing (1980a), only promoted shoot axes but not root development. This abnormality is one of the characteristics of precocious germination which is due to a fast transition between the late embryogeny stage to the germination stage without going through the mature and developmental arrest phase (Lelu et al. 1994a). Supplementing the germination medium with abscisic acid at 40 or 60 μM was shown to overcome this precocious problem in hybrid larch and other *Pinus* species (Lelu et al. 1994 a,b). In this study, adding ABA to the germination medium, did not restore the root formation of germinating somatic embryos. However, when activated charcoal was added to the germination medium, root formation in the somatic embryos was promoted. Thus, it was not clear if the lack of root formation of somatic embryos of *P. quinquefoilum* L. germinated on medium without charcoal was due to the precocious germination problem or to other problem such as the production of inhibitory substances by the cultured tissues which may be overcome by the supplement of activated charcoal.

In this study, plantlet recovery of *P. quinquefolium* L. was achieved through a two-stage process: the elongation of the shoot axes, followed by the formation of the root. A similar approach was also made by Arya et al. (1993) with *P. ginseng* C.A.

Meyer. The highest percentage of shoot axes elongation (70%) and plantlet formation (90%) in this study, were observed with half strength MS salts containing charcoal (1%) and NAA/2,4-D (5/4.5 μM) with charcoal (1%), respectively (Table 7 and Table 8).

In the previous study by Wang (1990), the frequency of somatic embryo formation was not indicated, although he achieved 30% plantlet recovery with IBA/NAA (2.5/0.5 μM). In my study, using seedling-derived leaf explants, the frequency of somatic embryo formation was 40% on NAA/2,4-D (10.0/9.0 μM) (Table 6) and plantlet recovery from embryos was 90% with NAA/2,4-D (5.0/4.5 μM) with charcoal (1%) (Table 8). Moreover, the regeneration process could be achieved within a 6 month period: 3 months for somatic embryo development (Table 6) and 3 months for plantlet recovery (Table 8).

In conclusion, this study has described a regeneration system for *P. quinquefolium* L. through somatic embryogenesis. This method is potentially useful for micropropagation of this plant, as well as for the production of artificial seeds. Moreover, with the availability of this method, it could enhance the plant improvement program of *P. quinquefolium* L. and permit studies on genetic transformation.

CHAPTER III

Agrobacterium - mediated transformation of North American ginseng (*Panax quinquefolium* L.)

3.1 Introduction

The development of crop cultivars with enhanced disease resistance has been a major objective for plant breeders since the beginning of plant breeding. Through the recent development of plant genetic engineering techniques, it is now possible to incorporate new traits into plants, provided that the genes responsible for these traits have been identified. Several sophisticated methods are now available to alter the genetic composition of plants (Potrykus 1990; Lal and Lal 1993). By far, *Agrobacterium*-mediated transformation is the most widely employed method since it provides stable integration of a well defined DNA segment in one or a few copies into the plant genome (Gheysen et al. 1992).

Natural defence mechanisms against infection by pathogenic microorganisms appear to have evolved in many plant species (Harms 1992; Kamoun and Kado 1993). One such mechanism is the production of chitinase enzymes which hydrolyze chitin, a component of fungal cell walls (Collinge et al. 1993). Based on a comparison of the predicted amino acid sequences, plant chitinases have been characterized and grouped into six classes (Meins et al. 1994). Class I chitinases are usually basic and vacuolar and have a highly conserved cysteine-rich domain of approximately 40 amino acids (Collinge et al. 1993). An extension of the C terminal consisting of seven amino acids is believed to be responsible for targetting the protein to the vacuole (Neuhaus et al. 1991). Class II chitinases are similar to class I (60-64% homology) but lack a cysteine rich N-terminal. These type of chitinases are generally acidic and targeted extracellularly. Class III chitinases have no sequence

similarity with class I and class II chitinases and can be either acidic or basic (Collinge et al. 1993; Punja and Zhang 1993). Class IV chitinases contain a cysteine-rich domain and a conserved main structure which is similar to class I chitinases but are significantly smaller in size (Meins et al. 1994). Class V chitinases have an amino acid sequence > 50% similar to the bacterial exo-chitinases from *Bacillus circulans*, *Serratia marcescens*, and *Streptomyces plicatus*, but no sequence similar to the class I-V (Meins et al. 1994).

Elevating the chitinase levels by introducing foreign chitinase genes into plants has been proposed as one way to improve plant resistance to fungal pathogens (Nitzche 1983). In fact, transgenic tobacco and canola plants, both expressing bean endochitinase class I, showed reduced fungal growth and delayed symptom development when infected with *Rhizoctonia solani* (Broglie et al. 1991). In contrast, there was no increased resistance to *Cercospora nicotianae* observed, from both transgenic *Nicotiana benthamiana* expressing an acidic class III chitinase from sugar beet (*Beta vulgaris*) (Nielsen et al. 1993), and *Nicotiana sylvestris* expressing tobacco class I (basic) chitinase gene (Neuhaus et al. 1991).

Agrobacterium-mediated transformation of ginseng to date has focused solely on Korean ginseng, with the objective being to elevate ginsenoside levels. Several investigators (Yoshikawa and Furuya 1987; Hwang et al. 1991 and Inomata et al. 1993 have established hairy root cultures of Korean ginseng (*P. ginseng* C.A. Meyer), after infection of root explants with *Agrobacterium rhizogenes*. At present, there are no reports of transformation of North American ginseng either for improving ginsenoside production or for increasing disease resistance.

The efficiency of transformation and production of transgenic plants depends on the establishment of optimal protocols for inoculation, and subsequent selection and regeneration of transformed cells (Draper et al. 1988). Using the established regeneration system for North American ginseng from the previous experiments, the

objective of this research was to develop procedures to introduce a foreign chitinase gene using *Agrobacterium*-mediated transformation by infection of root and mature leaf explants.

3.2 Materials and Methods

3.2.1 Plant materials.

3.2.1.1 Root explants.

For each transformation experiment, root explants from 4- to 5-year old field grown plants were sterilized as described previously (see Chapter II), and either directly infected or first pre-cultured onto MS medium supplemented with dicamba/kinetin (9.0/5.0 μM) for 1 or 2 weeks in the dark. Prior to infection with *Agrobacterium*, the explants were recut into segments (0.2-0.3 cm wide x 0.4 - 0.6 cm long). For each experiment, 10 - 16 segments were plated in each petri dish, with at least 10 replicate dishes.

3.2.1.2 Leaf explants.

Mature leaf explants originating from 4 year old field-grown ginseng plants were first sterilized as described in Chapter II and subsequently plated onto MS medium supplemented with NAA/2,4-D (9.0/10.0 μM) and dicamba/kinetin (4.5/5.0 μM). Infection was done directly after the leaf was disinfested or 2 weeks after being precultured on the above medium. Sixteen leaf segments of 0.2-0.4 cm^2 were placed in each petri plate, with at least 10 replicate dishes for each experiment.

3.2.2 Bacterial strains and plasmids.

For root explants, the *A. tumefaciens* disarmed strain EHA 105 containing the plasmid p1779C was used in this study (provided by Kay Lawton, Ciba-Geigy, Research Triangle Park, North Carolina). This vector contains both the neomycin

phosphotransferase - II (NPT - II) and cucumber acidic chitinase gene within the T-DNA border . *A. tumefaciens* disarmed strain EHA 105 containing plasmid pGA492-CHN was used for leaf explants. This vector also contains NPT II as a selectable marker and bean chitinase class I (courtesy of R. Broglie, DuPont Agric. Company).

Initially, the bacteria were grown overnight in 10 ml of LB medium (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, pH 5.4) containing 20 mg/l gentamycin at 29 ^{o}C. The next day, 1 ml of the overnight bacterial solution was resuspended in 25 ml Minimal Medium (MM) containing 20 mg/l gentamycin. On the third day, the bacterial suspension was spun at 2000 rpm for 10 min and the pellet was resuspended in MS medium (pH 5.4) to a final density of about 10^{8} bacterial cells/ml. Prior to transformation, acetosyringone (100 µM) was added.

3.2.3 Cocultivation and selection of transformants.

For root explants, infection was done by immersing the callused explants in the bacterial solution for 5 min., followed by rinsing with MS medium and finally blotting dry using sterile filter paper. The explants were placed on callus induction medium: MS with dicamba (9 µM) and kinetin (5 µM)) containing no antibiotics and incubated at 27^{o}C in the dark for 2 days of cocultivation. The explants were then transferred to a selective medium (same as callus induction medium) but supplemented with 50 mg/l kanamycin and 500 mg/l carbenicillin. Positive controls (root explants with no infection placed onto callus initiation medium only) as well as negative controls (root explants with no infection but placed onto selective medium) were included in each experiment.

For leaf explants, infection was done by immersing leaf segments for 3 min into the *Agrobacterium* solution. Different lengths of cocultivation (2 or 3 days) were tested for leaf explants. Following the transformation, leaf explants were placed onto two different media for cocultivation : MS medium supplemented with NAA/2,4-D

(10.0/9.0 μM) and dicamba/kinetin (4.5/5.0 μM). Two or three days after cocultivation, leaf explants were transferred to selective medium (same medium as above but containing kanamycin (50 mg/l) and carbenicillin (500 mg/l)). Positive controls (leaf explants with no infection placed onto callus induction medium without kanamycin and carbenicillin) and negative controls (leaf explants with no infection placed onto selective medium) were always included in each experiment.

All explants for the transformation experiment, were incubated in the dark at 24 - 26 ^{o}C and subcultured at monthly intervals onto the same medium for callus growth. Subsequent regeneration of these calluses into plantlets was carried through somatic embryogenesis as established in the previous experiment.

3.3 Results

3.3.1 Root explants

Upon inoculation with *Agrobacterium*, root explants from different periods of preculture (0, 1, and 2 weeks) prior to the transformation experiments showed different responses. The highest callus survival rate was 15.5% observed on 1 week precultured root explants, followed by 2.8% and 0% from 2 weeks and 0 week precultured root explants, respectively. The surviving calluses, were subsequently recut into 0.2 cm^2 segments and replated onto the selective medium (callus induction medium containing dicamba/kinetin (9.0/5.0 μM) and kanamycin 50 mg/l). These calluses continued to grow. In contrast, the root-derived calluses plated onto selective medium without prior infection with *Agrobacterium* (negative control), failed to develop.

Table 9. The effect of preculture of root explants of *Panax quinquefolium* L. before infection with *Agrobacterium tumefaciens* on the number of surviving explants.

Preculture duration	Surviving explants[a]
0 week	0%
1 week	15.5 %
2 weeks	2.8 %

[a] Rated 1 month after infection.

3.3.2 Leaf explants

Different media and different periods of cocultivation of leaf explants from 4-year old plants, gave different explant survival rates. Higher explant survival (28%) was achieved with 3 days cocultivation on medium with NAA/2,4-D (10.0/9.0 μM). With 2 days cocultivation on the same medium, 22.2% of mature leaf explants survived. On D1K1 medium, cocultivation periods of 2 and 3 days, gave rates of survival of 8.6% and 19.8%, respectively.

Preculture of mature leaf explants on medium D1K1 had no effect on the rate of survival of explants, whereas for medium N10D9, preculture of 2 weeks prior to the infection reduced the rate of survival of explants from 28% to 10.6%.

Table 10. The effect of preculture and different cocultivation periods for leaf explants from 4-year old *Panax quinquefolium* L. on the number of surviving explants.

	Cocultivation period	Surviving explants[a]	
		D1K1[b]	N10D9[c]
A.	No preculture		
	2 days	8.6%	22.2%
	3 days	19.8%	28.0%
B.	Preculture 2 weeks		
	3 days	18.6%	10.6%

[a] Rated 1 month after infection

[b] Dicamba/kinetin (4.5/5.0 μM)

[c] NAA/2,4-D (10.0/9.0 μM)

3.4 Discussion

It was observed that preculture of root explants had a positive effect in increasing the percentage of putatively transgenic calluses. The highest percentage of putatively transformed root-derived calluses was obtained with 1 week of preculture (15.5%). A positive effect of preculture was also observed with root explants of *Arabidopsis thaliana*. Three days preculture of root explants on callus induction medium, was observed to elevate the frequency of transformed calluses to 80% (Sangwan et al. 1992). de Jong et al. (1993) also recommended that the negative influence of cocultivation with *Agrobacterium* on *in vitro* plant regeneration could be minimized by separating the explant preparation such as cutting, from infection and cocultivation.

The highest percentage survival for leaf explants, was obtained without preculture, and with 3 days cocultivation period on medium with NAA/2,4-D (10.0/9.0 μM). According to Sangwan et al. (1992), competent cells for transformation of leaf explants with *Agrobacterium tumefaciens* are the dedifferentiating mesophylls cells, whereas for root explants, they are the dedifferentiating pericycle cells. Transformation 'competent' cells are cells within explants that have particular metabolic conditions which enable them to respond to certain physiological signals, such as wounding and phytohormones, and as a result, become more susceptible to infection and transformation by *Agrobacterium* (Sangwan et al. 1992). Competent cells of both root and leaf could be induced by either wounding and/or phytohormone. For leaf explants, but not for root explants of *A. thaliana*, wounding alone was sufficient to induce both cell activation and cell division. Preculture with phytohormone was required for root explants, but, not for leaf explants of *A. thaliana* (Sangwan et al. 1992).

Following cocultivation with *A. tumefaciens,* however, the regeneration capacity of putatively transformed calluses of both root and mature leaf explants was significantly reduced. Similar results were also observed with cowpea (*Vigna unguiculata* Walp) (Garcia et al. 1986; Perkins et al. 1987) and mustard (*Brassica juncea* L. (Mathews et al. 1990). One possible explanation of the suppressed regeneration capacity after infection with *Agrobacterium* is due to the fact that very often, the cells that are competent for transformation are not necessarily competent for regeneration (Potrykus 1990). Therefore, obtaining cells that are both competent for transformation and regeneration is very critical for establishing successful regeneration of transgenic plants. In this study, two types of explants, root and mature leaf were evaluated. However, regeneration of transgenic plants was not achieved. Regeneration of transgenic plants in these experiments was carried out through somatic embryogenesis. As suggested by Zimmerman (1993), the highest embryogenic potential of explants are within the first year of their development. Therefore, for future experiments, evaluating other type of explants, such as somatic embryos produced *in vitro*, seeds, seedling leaf explants, cotyledon and hypocotyl with different inoculation and cocultivation conditions is recommended. More knowledge of the relative susceptibility of different cells and tissues to transformation and how these putatively transformed tissues behave under selection and regeneration treatments would help develop strategies to optimize the transformation efficiency and the production of transgenic plants.

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